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Systemic drugs are one of the most potent means for controlling breast cancer. Their ability to kill cancer cells often depends, however, on the presence of appropriate physiological conditions. For instance, exciting recent results suggest that uptake and retention of 5-fluorouracil (5-FU) in tumors may be influenced by pH, in particular, the trans membrane pH gradient. This opens the possibility of predicting which tumors will show best response by measuring pH *a priori* or modulating tumor physiology to optimize tumor selectivity. We have developed a novel class of non-invasive NMR pH indicators and propose a novel second generation of enhanced indicators, which we will use to investigate breast tumor pH.

During the third year we have examined the use of novel ^{19}F NMR pH indicators in cultured tumor cells, perfused organs and initiated studies in vivo. The validity of ^{19}F NMR pH measurements of both intra and extra cellular pH has been further validated. We have initiated studies to predict the uptake and activity of 5-FU

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Introduction

Systemic drugs are one of the most potent means for controlling breast cancer. Their ability to kill cancer cells often depends, however, on the presence of appropriate physiological conditions. For instance, exciting recent results suggest that uptake and retention of 5-fluorouracil (5-FU) in tumors may be influenced by pH, in particular, the trans membrane pH gradient. This opens the possibility of predicting which tumors will show best response by measuring pH *a priori* or modulating tumor physiology to optimize tumor selectivity. We have developed a novel class of non-invasive NMR pH indicators and propose a novel second generation of enhanced indicators, which we will use to investigate breast tumor pH. Specifically, we will survey a series of diverse breast tumors in order to examine the specific correlation of 5-FU uptake versus pH gradient. In addition, we shall investigate the feasibility of manipulating pH in order to enhance breast tumor uptake.

We have three specific aims for this project:

Phase 1 Design, synthesis and evaluation of next generation molecules:
Based on our experience with 6-fluoropyridoxamine (6-FPAM) (1, 2), we have designed a second generation of enhanced pH indicator incorporating a trifluoromethyl group. These will provide three times greater signal intensity, improving the precision, and accuracy, of pH measurements *in vivo*. We will synthesize and characterize this new generation of improved ^{19}F NMR pH indicators.

Phase 2 Evaluation of a novel series of pH indicators *in vivo:*
Test trifluoromethyl pyridoxol in breast tumors *in vivo*. We believe the proposed pH indicators incorporating CF_3 reporter groups will offer considerable advantages over our current pH indicator 6-FPAM. However, should they fail to meet expectations (*e.g.*, due to toxicity or failure to cross cell membranes) then our current best reporter molecule (6-FPAM) will be used. These fluorine NMR pH indicators exhibit very strong NMR chemical shift response to changes in pH. They readily permeate cells providing simultaneous measurement of intra- and extracellular pH, and hence, the pH gradient.

Phase 3 Application of the new molecules to critical issues in breast cancer:
We will compare the regulation of pH gradients in two human and two rat tumor sublines that exhibit diverse therapeutic response (*viz.* mammary adenocarcinoma 13672- NF and PAM-CTX sublines and human breast tumors with differing metastatic potential, p53 and Her 2-neu expression),

Specific hypotheses to be tested include: a) that breast tumor pH may be measured accurately, and reproducibly, with useful temporal resolution using ^{19}F MR; b) that CF_3-pyridoxol will provide enhanced pH measurement in breast tumors, and c) that tumors exhibiting different therapeutic response exhibit significant differences in pH regulation. We believe this research will enhance the understanding of tumor pH dynamics, and promises a new prognostic tool to optimize therapy for individual breast tumors.

Body

During the third year both chemists continued to make progress in terms of learning and developing synthetic strategies and becoming familiar with in vivo NMR. Several possible alternate pH indicators were evaluated providing useful correlative measurements of pH response and structure activity relationships. These agents were attractive alternatives since the original synthetic strategies have presented greater hurdles than anticipated. However, these commercial agents suffered from poor solubility or poor chemical shift response or toxic. Thus, we continue to pursue the original synthetic goals. The primary NMR system for small animal research remained out of commission for several months in winter and spring and thus our schedule is delayed. We have requested and been granted a one year no additional cost extension to the grant and we expect to complete all the tasks during this additional time. During the third year several indicators molecules were tested in perfused organs and cultured cells to establish potential toxicity and intra cellular uptake. This has allowed us to identify CF3-POL as an indicator of extra cellular pH. Meanwhile, we have achieved our first examples of intra cellular penetration of the 6-fluoropyridoxamine indicators into several different tumor cells. Initial results have shown penetration into immortalized Chinese hamster ovary cells (CHO) and hepatoma cells. We have now established cell culture capacities in our own laboratory and received diverse breast cancer cells to pursue these critical investigations. We have initiated studies of uptake of both the pH indicators and chemotherapeutic drug (5-FU) in vivo and anticipate completing the proposed investigations during the one-year extension.

Statement of Work

Phase 1 Design, synthesis, and evaluation of enhanced ^{19}F NMR pH indicators:

Task 1 Months 1-6: Recruit and train post-doctoral fellow in synthesis of fluorinated vitamin B6 analogs

Completed year 1 and 2, thought chemist (Dr. Jianxin Yu) continues to refine synthetic studies to optimize product yields and new synthetic strategies.

Task 2 Months 3-9 Synthesize CF_3-pyridoxol

Completed years 1 and 2, manuscripts in preparation.

Task 3 Months 6-12 Synthesize CF_3-pyridoxol derivatives modified at 4, 5 and 2 position

Completed in years 1 and 2

Task 4 Months 3-12 Characterize new pH indicators, *e.g.*, ^{19}F NMR titrations, high resolution mass spectrometry, ^{1}H, ^{19}F and ^{13}C NMR structural analysis

We have performed additional calibrations and titrations on an alternate class of ^{19}F NMR pH indicator, which may be useful in vivo (Appendix item 1). In particular the results provide a database to assist in evaluating chemical, structural, and spectral characters useful for a ^{19}F NMR pH indicator.

Task 5 Months 6-12: Evaluate molecules in plasma and whole blood

Further tests have been conducted to validate the results of years 1 and 2 and examine the new agents assessed under task 4. (Appendix 2)

Task 6 Months 9-15: Scale up synthesis of most promising molecules

CF_3-pyridoxol was produced in 300 mg quantity. Further scale up, as needed.

Task 7 Months 9-18: Evaluate most promising molecules in perfused heart model
Dr. Cui was recruited during year 2 and has become familiar with in vivo high field NMR spectroscopy and imaging. pH reporters have been tested both in perfused hearts and perfused kidneys (Appendix 3). Tests in the heart provide a very effective indication of any acute toxicity since the heart rate and developed pressue are readily observed.

Task 8 Month 12: Prepare reports and manuscript.
Report provided. Further manuscripts in preparation

Phase 2 Evaluation of optimal pH indicator *in vivo*:

Task 9 Month 9-18: Evaluate best molecule in breast tumor subline 13762NF (6 tumors)
i: Surgically create pedicles for tumor implantation
ii: Implant tumors and allow to grow to 1 cm diameter
iii: Examine pharmacodynamics of pH indicators *in vivo*; measure baseline pH; verify validity of measurements using ^{31}P NMR and electrodes.

Tumors have been prepared. Dr. Cui has initiated studies in vivo in both animal control and tumor bearing rats. Some tests have shown a lack of ^{19}F NMR signals in tumors in vivo and Dr. Cui has conducted several tests in cultured tumor cells to assess the extent of pH reporter uptake. Results are summarized in Appendix 4. In vivo experiments were further delayed because the 4.7 T MR system was out of commission for several months during the winter and spring of 2002. The system has now been upgraded to a Varian INOVA unity system and shows good stability and enhanced sensitivity. Dr. Cui found that the new gaseous anesthesia protocol can cause potential problems since isoflurane produces 2 NMR visible peaks in the range of some pH indicators. Thus, judicious caution is required to avoid overlap of signals. The signal from CF3-POL is well removed from the isoflurane.

Task 10 Months 18-34: Synthesis gram quantities of optimal molecule for *in vivo* use.
Synthesis of 6-FPAM and CF3-POL have been scaled up to facilitate in vivo investigations in rats and mice

Task 11 Months 19-21 5-FU pharmacokinetics in rat small breast tumors as function of ΔpH (6 tumors for each of the rat mammary adenocarcinoma 13672 sublines NF and LPAM CTX)

We have initiated tests of the uptake and biodistribution of 5-FU in tumor bearing rats.

Tasks 12–18
Will be undertaken on an accelerated basis during coming year. We have requested and been approved for a 1 year no additional cost extension during which time we expect to be able to complete all the original goals successfully.

Key Research accomplishments

- Evaluation of titration curves for CF_3-pyridoxol pH indicator in solution and characterization in whole blood and perfused organs
- First demonstration of the entry of 6-fluoropyridoxamine into cancer cells permitting non-invasive measurement of transmembrane pH gradient.
- Examination of uptake of 5-FU in tumors.

Reportable outcomes

Three papers describing work supported by this grant were presented at International conferences during 2002. Two were oral presentations at the International Society of Magnetic Resonance in Medicine. These papers are selected by peer review and oral presentations are reserved for the "best" and most exciting work. A third paper was presented as poster at the Era of Hope Meeting.

1 "A novel NMR reporter molecule for transmembrane pH gradients: para-fluoro-ortho-nitrophenol". W. Cui, P. Otten, M. Merritt, and **R. P. Mason**, ISMRM 10th Scientific Meeting, Honolulu, Hawai'i, USA 18 - 24 May, 2002

2 "Gene reporter molecules: a novel approach revealing β-galactosidase activity". **R. P. Mason**, P. Otten, Y. Li and K. Koeneman, ISMRM 10th Scientific Meeting, 384, Honolulu, Hawai'i, USA 18 - 24 May 2002

3 "Breast tumor pH: design and evaluation of novel reporter molecules", **R. P. Mason**, P. Otten, W. Cui & J. Yu, Era of Hope, Orlando, P49-10, Sept. 2002.

Conclusions

- CF_3 analogues of F-pyridoxine pH indicators can be synthesized and, as predicted, show a sensitivity ~ 1.5 ppm. ^{19}F resonances are sufficiently narrow to allow useful NMR discrimination of pH.
- Synthesis of the fluoropyridoxol is complex and requires highly skilled organic chemist. We thus evaluated several "off the shelf" commercial agents as possible alternate pH indicators. While some had interesting pH sensitivity, several were highly toxic or exhibited poor water solubility. These results justify the synthetic undertaking proposed in this grant.
- CF3-POL does not enter cells and may be useful as an indicator of extra cellular pH (pHe). This may be very important, since intra cellular pH can often be determined based on endogenous inorganic phosphate by 31P NMR. pHe has been largely elusive and new indicators are need. We will continue to evaluate the utility of this agent,
- For the first time we have observed 6-fluoropyridoxamine enter cells. This will allow us to complete the remaining tasks of the project

References

1. He, S., Mason, R. P., Hunjan, S., Mehta, V. D., Arora, V., Katipally, R., Kulkarni, P. V., and Antich, P. P. Development of Novel ^{19}F NMR pH Indicators: Synthesis and Evaluation of a Series of Fluorinated Vitamin B_6 Analogs, Bioorg. Med. Chem. *6:* 1631-9, 1998.
2. Mason, R. P. Transmembrane pH gradients *in vivo*: measurements using fluorinated vitamin B6 derivatives, Curr. Med. Chem. *6:* 481-499, 1999.

Appendix 1

^{19}F NMR titration curves of diverse potential reporter pH indicator molecules

Commercial trifluoromethyl molecules

Materials: All chemicals were bought from Lancaster or Aldrich. pH meter 440 was used to measure pH. NMR was performed on Varian 600 spectrometer.

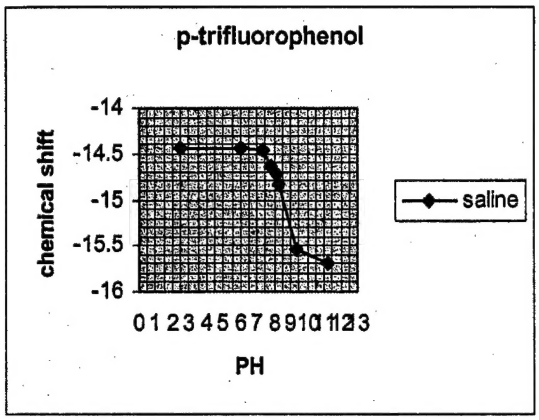
Method: The chemical compound was dissolved in saline, 0.2N HCl and 0.25N NaOH was added to change pH. NMR was acquired at 25 °C, and pH was measured by electrode. TFA-Na in capillary was used as a chemical shift standard.

Results:

a. p- trifluorophenol, b. 2-chloro-3-trifluoromethyl-phenol, c. 0-trifluoromethyl-phenol, **d**. 4-trifluoromethyl-2-pyrimidinol, e. 6-trifluoromethyl-4-pyrimidinol, f. 2-nitro-4-trifluoromethyl-phenol, g. 4-nitro-3-trifluoromethyl-phenol, h. 5-trifluoromethyl-2-pyridinol, i. α,α,α-trifluoro-m-cresol, j. 2-chloro-5-trifluoromethyl-phenol,

compound	pH(elec) change	Chemical shift change	pKa	Note
a	2.51~11.23	-14.439~-15.694	8.5	When pH>8.1, it shows two peaks
b				Three signals, difficult to tell
c	1.91~10.66	13.63~14.03	7.92	
d	1.84~10.53	4.09~5.15	6.83	Solubility bad
e	1.88~10.3	4.31~5.17	6.74	Solubility bad
f	2.05~9.65	13.49~14.52 11.5~11.47	5.46	Two signals around –11ppm and –13ppm
g	2.27~9.65	15.27~15.06 13.94~14.53	6.0	When pH is changed, two signals show.
h	2.2~11.28	13.09~14.99	9.5	
i	1.95~8.74	13.02~13.11	8.3	
j	1.6~12.35	13.09~13.38	6.5	Two signals show when pH<7.8

a



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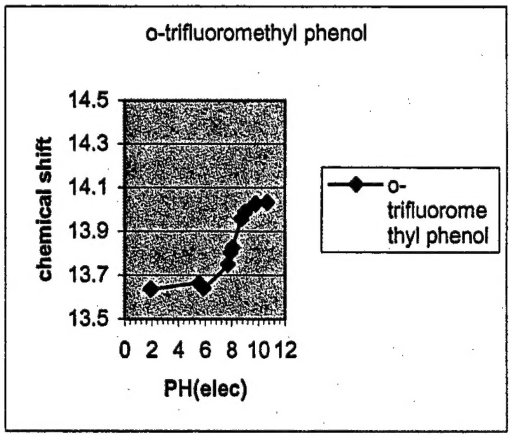
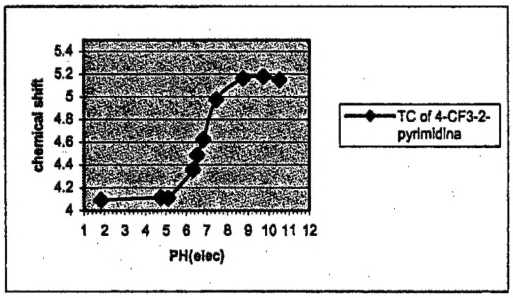
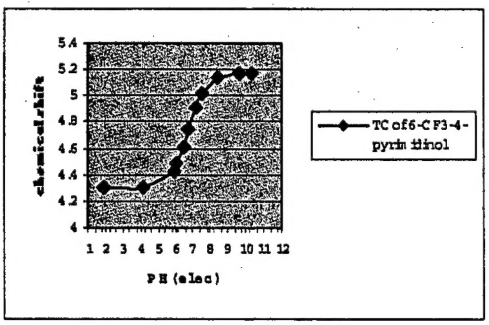


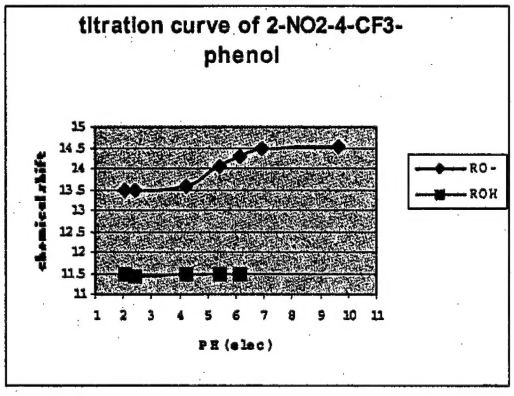
d 4-trifluoromethyl-2-pyrimidinol,



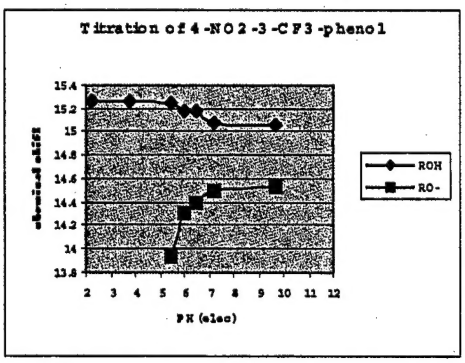
e 6-trifluoromethyl-4-pyrimidinol



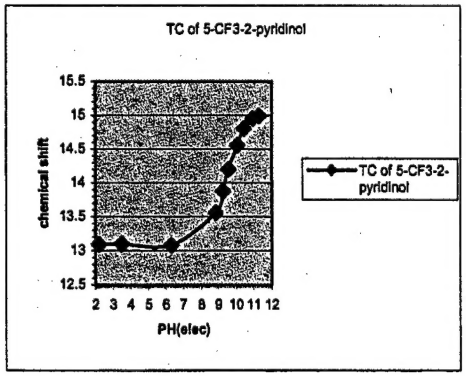
f 2-nitro-4-trifluoromethyl-phenol



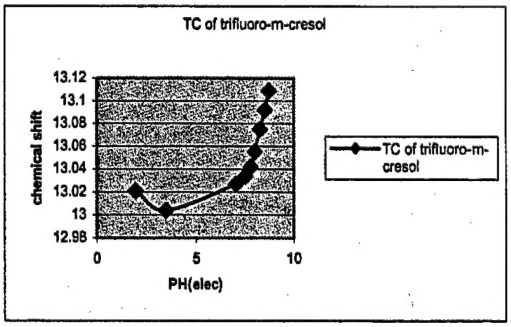
g. 4-nitro-3-trifluoromethyl-phenol



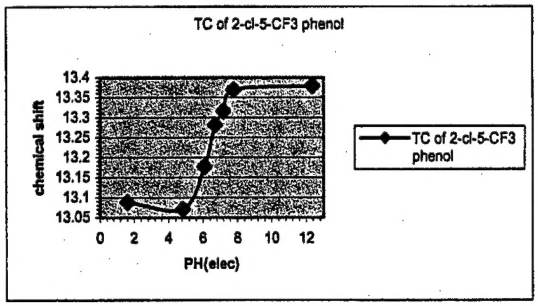
h. 5-trifluoromethyl-2-pyridinol



i α,α,α-trifluoro-m-cresol,



j 2-chloro-5-trifluoromethyl-phenol,

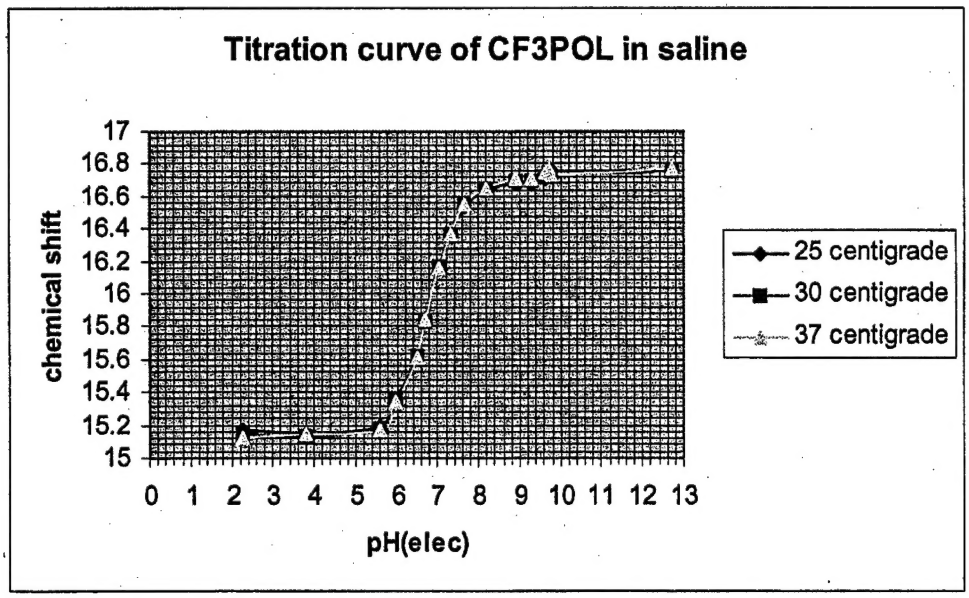


Conclusion: The above chemicals are not suitable for pH indicators: the pKa of compound **a, f, g, h, i** is not close to physiological condition; compound **b, f, g, j** have two or more signals, which is confusing; the solubility of **d** and **e** is poor; the chemical shift difference of compound **c** is small.

6-trifluoromethyl pyridoxol (CF_3-POL) In vitro

Materials: NMR was performed on Varian 9.4T spectrometer. pH was measured on pH meter 440.

Results:
Titration curves: pKa=6.82 δacid=15.12 ppm, δbase=16.76



Conclusion: the titration curves at different temperatures are identical.

Appendix 2

Assessment of ^{19}F NMR pH reporters in blood plasma and cells

Para fluoro ortho nitro phenol (PFONP)

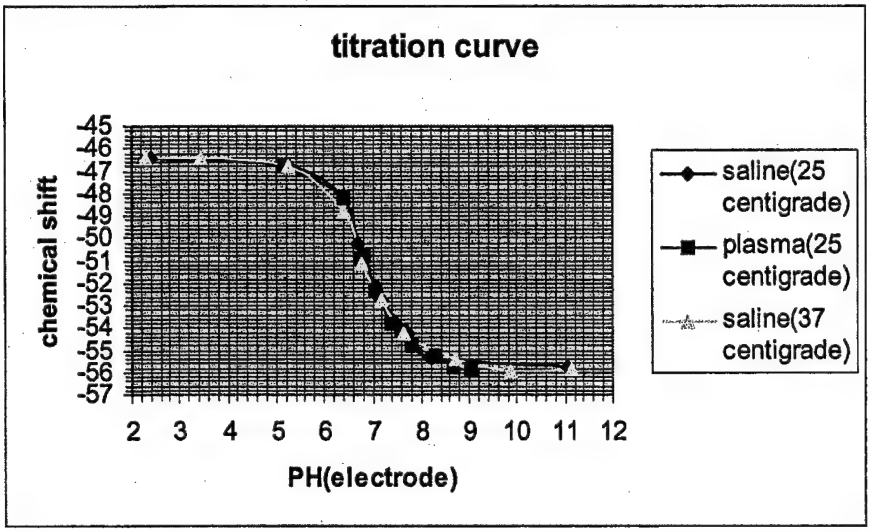
Materials: PFONP was purchased from Lancaster. 6-fluoropyridoxol (FPOL) was synthesized by Dr. Pieter Otten. Fresh rabbit blood was provided by Anca Constantinescu. NMR was performed on Varian 14.1T spectrometer. pH was measured using pH meter 440. Whole blood was centrifuged at 6500 RPM to get red cells and plasma.

Method: PFONP and FPOL were added into saline, whole blood, plasma, and red cells, NMR was acquired at different centigrade. 0.2N HCl and 0.25N NaOH was added to change pH. TFA-Na is a reference.

In order to diminish the signal of 2,3-DPG, fresh blood was kept at 37 °C for 5 hr, or was centrifuged and washed with saline repeatedly.

Results:

1. Titration curves in saline and plasma



Conclusion: we found that PFONP has a single ^{19}F-NMR signal, which is highly sensitive to pH exhibiting a range of 9.3 ppm between acid and base as shown in the titration curve. The pKa=6.85 and Henderson-Hasselbalch coefficients are δacid= -46.44 ppm, are δbase= -55.75 ppm with respect to TFA=0ppm. The titration curves were found to be identical in saline or plasma.

2. Samples were prepared and acquired at 25 °C.

The comparison of pH measured by Electrode and NMR

sample	pH(electrode)	δ(ppm) of PFONP	pH calculation	δ(ppm) of FPOL	pH(calculation)
PFONP in whole blood	7.45	-52.179 -53.617	7.05 7.35		
PFONP in red cell	7.41	-52.361 -53.796	7.09 7.42		
PFONP in plasma	7.40	-53.799	7.42		
PFONP +FPOL in	7.66	-52.676 -54.209	7.15 7.55	-10.93 -11.59	7.29 7.55

whole blood					
PFONP +FPOL in red cell	7.66	-52.384	7.10	-10.928 -11.634	7.29 7.61
PFONP +FPOL in plasma	7.68	-54.275	7.57	-10.743 -11.452	7.20 7.49

Conclusion: When PFONP was added to whole rabbit blood, two signals attributed to intra and extra cellular compartments were rapidly observed at –52.18 ppm and –53.62 ppm, corresponding to pHi=7.05 and pHe=7.35, and an electrode showed pH=7.45. When PFONP was added to red cells, the intracellular signal is much bigger than extracellular signal, corresponding to pHi=7.09, pHe=7.42, and an electrode showed pHe=7.41. When 6-fluoropyridoxol was added to whole blood and red cells as an alternate pH indicator, the two signals of PFONP in whole blood were at –52.68 and –54.21 ppm, attributed to pHi=7.16 and pHe=7.55, the signals of 6-fluoropyridoxol indicated pHi=7.29 and pHe=7.55, electrode showed pHe=7.66.

3. The comparison of ^{31}P and ^{19}F

Blood	pH(elec)	^{31}P						δ(PFONP)	pH(cal)	δ(FPOL)	pH(cal)
		Pi	pH Calc.	2-DPG	pH Calc.	3-DPG	pH calc				
fresh	7.68	2.98	7.77	3.27	7.26	4.04	7.25	-52.53 -54.65	7.12 7.68		
Kept at 37 degree for 5 hr		2.72	7.18					-53.15	7.27		
Kept at 37 degree for 5 hr		2.75	7.21					-53.13	7.26	-10.81	7.24
Red cells washed with saline		2.77	7.23					-54.66	7.73		
Red cells washed with saline		2.85	7.31					-54.665	7.74	-10.89 -11.65	7.28 7.55

4. The spin-lattice relaxation time of PFONP in blood and saline

T1 in saline: 3.6±0.06 s
T1 in fresh whole blood: 0.95±0.07 s

In cells:

Materials: Dr. Sophia Ran provided breast cancer cells. TK, CK, wt cells, and E.coli were provided by Dr. Zhenyi Ma.

Method: For breast cancer cells, TK, Ck, and wt cells, PFONP was added to cells, acquire data at 37 °C. Alternately for TK cells, 100 mg PFONP, dissolved with DMSO was added to culture medium, incubated with cells for 24 hr.

Results:

cells	concentration	Dosage of PFONP	signal
Breast cancer cells	5*10^8	5 mg	One signal
TK	2.8*10^7	5.9 mg	One signal
CK	301*10^7	5.8 mg	One signal
Wt	3.9*10^7	5.7 mg	One signal
E.coli			:one signal

TK cells: Almost all cells were dead after incubation with PFONP as judged by microscopy.

Conclusion: the toxicity of PFONP lysed cells.

6- Fluopyridoxamine in cells

Materials: E. coli and CHO, wt, ck, tk cells were provided by Dr. Zhenyi Ma.

Method: a: Add FPAM directly into E.coli and cells.
b. 100 mg FPAM dissolved with water/DMSO, incubated with cells for 12 hr.

c. Add FPAM directly to the cells, get the spectrum, centrifuge the sample, get the supernatant and cells in which buffer was added.
d. Add FPAM incubated with cells for 1 hr, then centrifuge, wash cells, add buffer.

cells	concentration	method	FPAM (mg)	δ(ppm)	pH(cal)	pH(elec)	
E. coli		a	5.4				
CK		b		-3.90 -19.1			Half of the cells died.

CK	4.3*10^7	a	6.0	-15.67 -17.80	7.31 7.84	8.03	
wt	1.0*10^7	a	6.2	-15.81 -17.84	7.34 7.86	7.98	
		Add FPOL to the cells		-14.16 -16.33	7.04 7.45	7.58	FPOL -11.19 (7.40)
TK		Add FPOL				7.35	-10.94 (7.30)
			5.5	-14.11 -16.34	7.02 7.45	7.51	-11.2 (7.40)
TK	6.5*10^7	c	6.0	-14.16 -15.04	7.04 7.20	7.09	
				-17.43	7.68	7.86	Cells and buffer
				-17.59	7.77		supernatant
CHO	9*10^6	c	4.7	-11.66 -16.32 -18.11	6.57 7.45 7.97	8.13	
				-18.33	8.08	8.49	Cells and buffer
wt	3*10^7	d	6.0	-5.15 -17.87	7.87	8.27	

Conclusion: From the above data, it appears that the two peaks both come from FPAM.

Appendix 3

Evaluation in perfused organs to assess toxicity and cell penetration
Perfused heart:

Materials: Fisher 344 rats were used.

Method: Rats were anesthetized and hearts excised. Retrograde perfusion was performed with recycled phosphate-free, modified Krebs-Henseleit buffer oxygenate with carbogen at 37 °C under a pressure of 100 cm H_2O. PFONP was put into recycling perfusate.

Results:

1. when 40 mg PFONP was added to 400 ml perfusate at r.t., it only partially dissolved. A filtrate was prepared and added to perfusion buffer. BP=50 mmHg, beating rate= 180/min. TFA-Na in capillary was put into the 20 mm NMR tube as reference. ^{31}P and ^{19}F spectrum was acquired. No any signal of PFONP.
   More PFONP was added at 37 °C until saturation, the heart stop beating immediately.
2. Another rat, BP=100 mmHg, beating rate=240/min. Saturation solution of PFONP at 37 °C was recycled, the heart stop beating immediately, then dilute the perfusate, the heart began to beat slowly. BP=10~20 mmHg. Get fluorine signal at –54.74 ppm (pH=7.76), pH(elec)=7.80.

Conclusion: The toxicity of PFONP is excessive. Decreasing dosage of PFONP, no signal can be obtained.

FPAM

Method: Same as that of PFONP. 100 mg FPAM was dissolved with 200 ml perfusate.

Results:

	Chemical shift	pH(cal)	pH(elec)
Pi	2.54	7.33	8.31
FPAM	-17.26 -18.38	7.67 8.2	8.31

CF3-POL

Materials: Fisher 344 or Copenhagen rats were used.

Method: Rats were anesthetized and heart excised. Retrograde perfusion was performed with recycled phosphate-free, modified Krebs-Henseleit buffer oxygenate with carbogen at 37 °C under a pressure of 100 cm H_2O. 30 mg CF3-POL was put into 150 ml perfusate to recycle.

Results: Only one signal at 16.41 ppm, pH(cal)=7.39, pH(elec)=7.28
Conclusion: CF3-POL does not penetrate cells in pefused organ.

Appendix 4

In vivo

Materials: Copenhagen rat with AT1 tumor and DMSO were provided by Anca. Self-made 2 cm $^{19}F/^{1}H$ tunable coil is used.

Method: PFONP was partially dissolved with saline and DMSO, injected by i.p or i.t. In order to increase the solubility, NaHCO3 was added to adjust pH to 7.99. TFA-Na was injected (i.p) as a standard.

Results:

PFONP	Sol vent	administration	signals	note
67 mg	DMSO	i.p	TFA isofluorane	
130 mg	Saline/DMSO	i.p	Died in 5 min	
10 mg	Saline/DMSO	i.t.	PFONP	
107 mg	Saline/DMSO/NaHCO3	i.p	Died in 5 min	

Conclusion: The solubility of PFONP is very bad. If it is dissolved with DMSO only, it is difficult to absorb, if the solubility is increased by adding base, the toxicity is too big.

6-Fluoropyridoxamine (FPAM)

In vivo

Method: FPAM was dissolved with saline or saline/DMSO, and was injected by i.p or i.v to rats with tumor or without tumor. Tumor or leg was put into coil to acquire ^{19}F signal.

Results:

1. 57 mg FPAM dissolved with saline injected I.V., put leg into 2 cm $^{19}F/^{1}H$ tunable coil. In 17 min, two peaks showed at –13.75 ppm (pH=6.97) and –14.22 ppm (pH=7.05).

Conclusion: FPAM was detected in vivo, but further tests are required to prove the two signals from I.V. injection come from FPAM and not signal of isoflurane.

CF3-POL

Materials: Fisher 344 rat with breast tumor and Copenhagen rat with prostate tumor were provided by Anca. CF3-POL was provided by Dr. Pieter. Otten.

Methods: Rats was anesthetized with ketamine. 160 mg/kg TFA-Na was injected by i.p. CF3-POL was dissolved by saline/DMSO, i.p.

Results:

Rats	Tumor size	CF3-POL	Chemical shift	pH(cal)	Note
Copenhagen	2.4*3.1*1.8	77.5 mg	16.42ppm	7.40	In 30 min, signal shows, it is still there in 90 min. Total acquisition time is 17min
Fisher 344	1.8*1.0*2.0	49.1 mg	16.33 ppm	7.27	The same as above

Conclusion: CF3-POL can be observed from tumor, and its solubility is better than other pH indicator.

BREAST TUMOR PH: DESIGN AND EVALUATION OF NOVEL REPORTER MOLECULES

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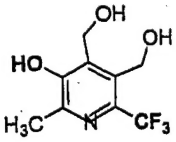
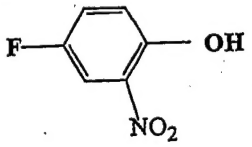
Many factors impact the efficacy of cytotoxic therapy for breast cancer. Local acidity (pH), and in particular, cellular transmembrane pH gradients can influence the distribution of therapeutic drugs. Historically, no satisfactory methods existed to measure transmembrane pH gradients *in vivo*. We have investigated new approaches using magnetic resonance reporter molecules and have identified two novel classes of agent.

6-(trifluoromethyl)pyridoxine (CF_3-POL; a vitamin B6 analogue) exhibits a chemical shift of 1.6 ppm between acid and base conditions and has a pKa = 6.7 making it ideal for investigations of breast cancer. Initial tests show that it provides reliable pH measurements in whole blood. The CF_3 group provides a three-fold gain in signal to noise over our previous prototype agents [*Curr. Med. Chem.* 6, 481 (1999)]. Unfortunately, CF_3-POL does not penetrate cancer cells and is restricted to providing estimates of extra cellular pH only. This can itself be valuable, but we are also exploring methods of stimulating cellular penetration and surveying various cell types for uptake characteristics.



CF_3-POL



PFONP

Para-fluoro-ortho-nitrophenol (PFONP) represents a novel class of pH sensor molecule. The single ^{19}F NMR signal exhibits a chemical shift range of 9.3 ppm between acid and base and pKa = 6.85. Upon addition to whole blood, two signals attributed to intra- and extra-cellular compartments were rapidly observed, *e.g.*, –52.68 and –54.21 ppm corresponding to pHi = 7.16 and pHe = 7.55. We are initiating studies of pH in breast tumors with this novel indicator. PFONP offers additional intriguing opportunities: it is a fluorinated analogue of the aglycone in ONPG (orthonitrophenol) the classical test reagent for β-galactosidase activity in histology. We have synthesized the corresponding glycoconjugate (PFONPG) and it is indeed a very sensitive reporter for enzyme activity.

The new reporter molecules will be valuable for assessing the relevance of tumor pH. Such measurement may facilitate improved therapeutic outcome based on the characteristics of a specific tumor. PFONPG opens the possibility of monitoring gene expression *in vivo* and interrogating local pH at sites of gene activity, which may impact the field of gene therapy.

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A novel NMR reporter molecule for transmembrane pH gradients: para-fluoro-ortho-nitrophenol

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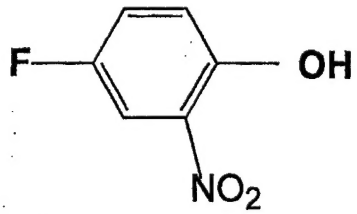
pH regulation is critical in many disease states; in particular it is thought to impact the efficacy of various cytotoxic therapies for cancer. Even more significant may be transmembrane pH, since this influences the distribution of weakly acidic or basic drugs. We have now identified para-fluoro-ortho-nitrophenol as a promising new sensor for measuring transmembrane pH gradients. We present titration curves together with initial measurements in solution and whole blood showing the veracity of this new approach.

Introduction

Many diseases generate perturbations in tissue physiology revealed by deficits in perfusion, oxygenation and pH. NMR provides a non-invasive means to monitor these parameters and dynamic changes in response to interventions. Previously, derivatives of vitamin B6 (6-fluoropyridoxol; FPOL) have been proposed for measuring transmembrane pH in blood, perfused heart and tumors (1), but synthetic preparation is laborious. Para-fluoro-ortho-nitrophenol (PFONP) is readily available commercially and presents structural characteristics compatible with a useful pH sensor molecule. We have now evaluated PFNP and present data showing its efficacy as an NMR reporter molecule.

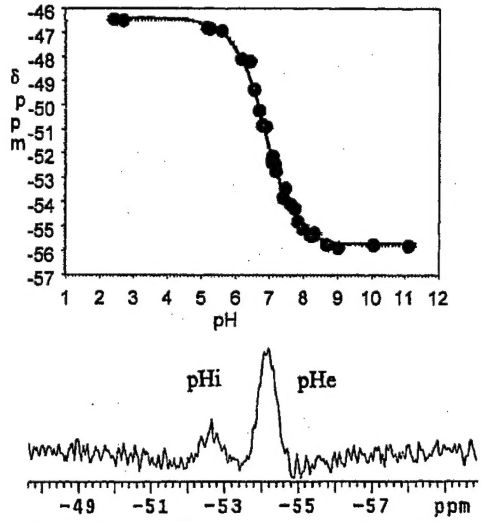
Materials and methods



PFONP was obtained from Aldrich. NMR characteristics and titration curves were assessed at 383 and 596 MHz at 25 °C or 37 °C in saline and plasma. Samples were added to fresh whole heparinized rabbit blood together with sodium trifluoroacetate (TFA) as a chemical shift reference and 6-fluoropyridoxol as an alternate pH indicator.

Results

PFONP has a single ^{19}F NMR signal in water. There are small H-F couplings, but these are generally not resolved in biological samples. The ^{19}F signal is highly sensitive to pH exhibiting a range of 9.3 ppm between acid and base as shown in the titration curve. The pKa = 6.85 and the Henderson Hasselbalch coefficients are δ_{acid} –46.44 ppm, δ_{base} –55.75 ppm with respect to TFA = 0 ppm. Even at neutral pH there is no excessive line broadening, though the water solubility is substantially lower under acidic conditions.



Partial 564 MHz ^{19}F NMR spectrum from whole rabbit blood containing 6 mg PFONP acquired in 2 mins.

The titration curves were found to be identical in saline or plasma. Upon addition to whole blood, two signals attributed to intra- and extra-cellular compartments are rapidly observed, *e.g.*, at –52.68 and –54.21 ppm corresponding to pHi = 7.16 and pHe = 7.55, as shown in spectrum above. Co-addition of 6-fluoropyridoxol indicated pHi = 7.29 and pHe 7.55 and an electrode showed pHe = 7.66. Signal assignment was confirmed by centrifuging the blood to separate cells and plasma components. T1 in whole blood was in the range 0.9 to 1.5 s.

Discussion

While many pH indicators have been proposed for NMR, the greatest hurdle is generally achieving cellular penetration in order to provide the trans membrane pH gradient based on a single reporter molecule observed in a homo nuclear experiment. PFONP shows pH sensitivity competitive with the best molecules reported hitherto and its ready commercial availability is attractive. Since the phenol is a weak acid, it must be added judiciously to biological samples so as not to perturb the intrinsic pH. Toxicity is in the range 400 mg/kg in mice. We are initiating studies to explore the range of applications for this novel indicator.

Reference

1 Mason, R. P., *Curr. Med. Chem.* **6**, 481, 1999

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Gene reporter molecules: a novel approach revealing β-galactosidase activity

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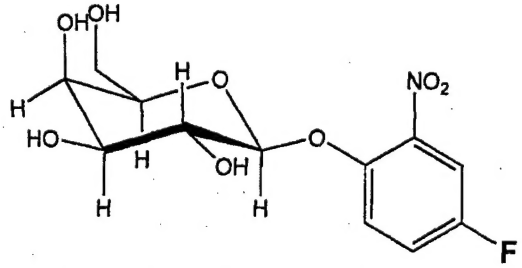
Gene therapy is generating increasing interest. However, a major issue is the success of targeting the gene to tissue of interest, the distribution of the gene, its activity and longevity of action. To this end various strategies have been proposed to detect gene expression. We present a novel concept: ^{19}F NMR detection of β-galactosidase activity based on the chemical shift change accompanying cleavage of the enzyme substrate para- fluoro- ortho- nitro- phenyl β-D-galactopyranoside. Here, we report the MR characteristics of this novel reporter molecule together with examples of its application *in vitro* and *in vivo*.

Introduction

The activity of most therapeutic genes is not directly detectable. A powerful tool is the incorporation of a tandem reporter gene, which reveals activity of genes of interest. Historically, the most popular reporter gene has been lac-z, which generates β-galactosidase (β-gal). Numerous biochemical assays are available to detect β-gal activity, but they have been limited to histology or *in vitro* assays. Recently, a ^{1}H MR contrast reporter molecule was presented (1), but this bridged cyclic paramagnetic agent requires complex synthesis, fails to penetrate cells and is a poor substrate for the enzyme. We reasoned that introduction of a fluorine atom into the traditional biochemical substrate ortho-nitro-phenyl galactopyranoside (ONPG), could provide a novel enzyme activity sensor (*viz.* gene reporter) with minimal perturbation to a well proven substrate.

Materials and methods



Para- fluoro- ortho- nitro- phenyl β-D-galactopyranoside (PFONPG) was synthesized using the methods of Yoon *et al.* (2). Following purification, the molecule was assessed at 9.4 and 14.1 T in various solutions, whole rabbit blood and cultured prostate tumor cells.

Results

PFONPG was isolated in good yields and found to have a single ^{19}F NMR signal at –42.66 ppm (with respect to sodium trifluoroacetate). The molecule was stable in water and whole rabbit blood for a period of hours.

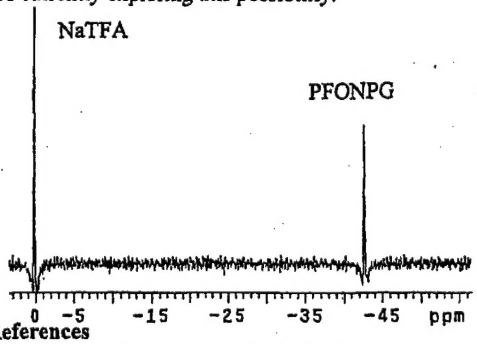
Addition of β-gal (aspergillus oryzae, Sigma) led to rapid cleavage liberating the aglycone, which appeared as a new peak ~ 5 ppm upfield, accompanied by color development. Incubation of PFONPG with cultured human prostate cancer cells (PC3 and C4-2) showed no enzyme activity. However, when these cells were infected overnight with adenovirus carrying the β-gal gene driven by CMV promoter, gene activity was revealed by liberation of the aglycone. Most significantly, the rate and extent of activity were in line with expected levels of gene expression.

Discussion

Introduction of the fluorine atom into the classical biochemical reagent ONPG provides a novel NMR reporter molecule to detect β-gal activity. Given the well known promiscuity of β-gal, diverse substrates could be developed, but maintaining close structural similarity to existing indicators may be most appropriate. The ^{19}F resonance of PFONPG does show small F-H coupling and ^{1}H decoupling could improve the signal to noise ratio. However, the coupling is generally not seen in biological samples due to inherent line broadening.

^{19}F NMR has the great advantage that there is essentially no background signal in tissue. Moreover, cleavage of the PFONPG provides definitive indication of enzyme activity revealed by the change in chemical shift. Nonetheless, studies may be limited to spectroscopy by the concentrations achievable *in vivo*.

The approach presented here, adds to the choice of methods for investigating gene transfection. Furthermore, the aglycone PFONP chosen here shows intrinsic pH sensitivity raising the intriguing possibility of determining local pH at the site of β-gal activity. We are currently exploring this possibility.



References

1. Louie, *et al. Nature Biotechnol.* **18**, 321, 2000
2. Yoon, *et al. Bull. Korean Chem. Soc.* **17**, 599, 1996

Supported by NCI P20 CA86354 and DAMD17-99-1-9381